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(58) Field of search
C3H

(54) Wound healing compositions

(57) A human platelet cell proliferation factor (HPPF) has been isolated. This platelet factor is characterized as an acid stable, thermostable peptide having an apparent molecular weight in the range of 8,000 to 12,500 and an isoelectric point in the range of from 3.5 to 5.5. In combination with a co-factor selected from cell growth factors, cell attachment factors and plasminogen activators, and particularly epidermal growth factor (EGF), HPPF promotes the healing of wounds.

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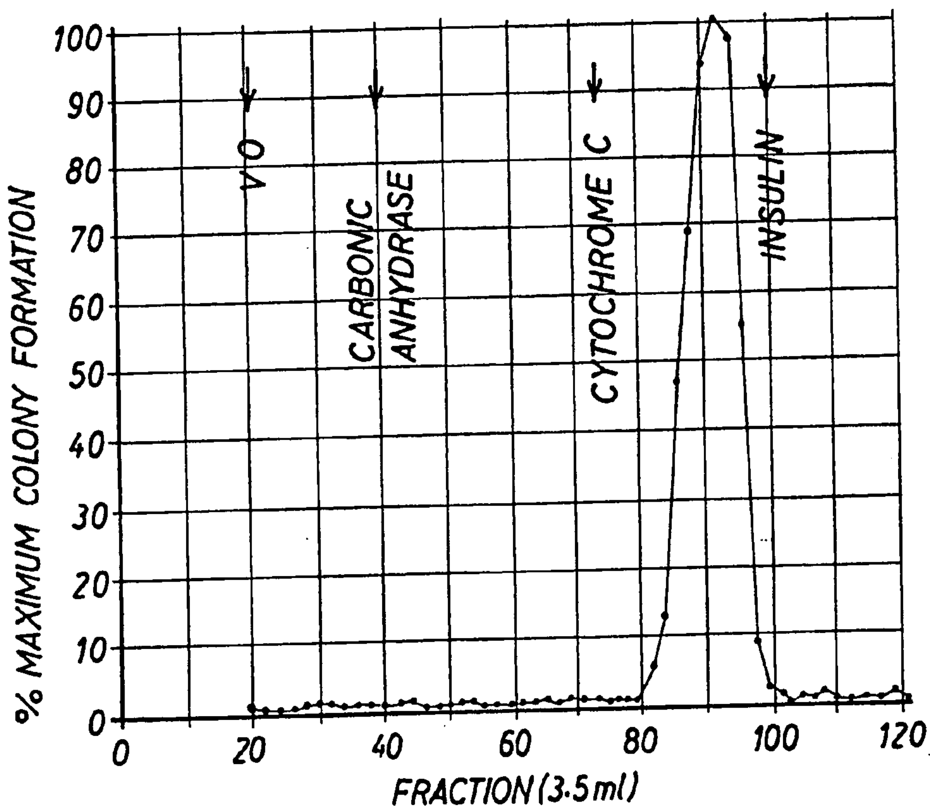
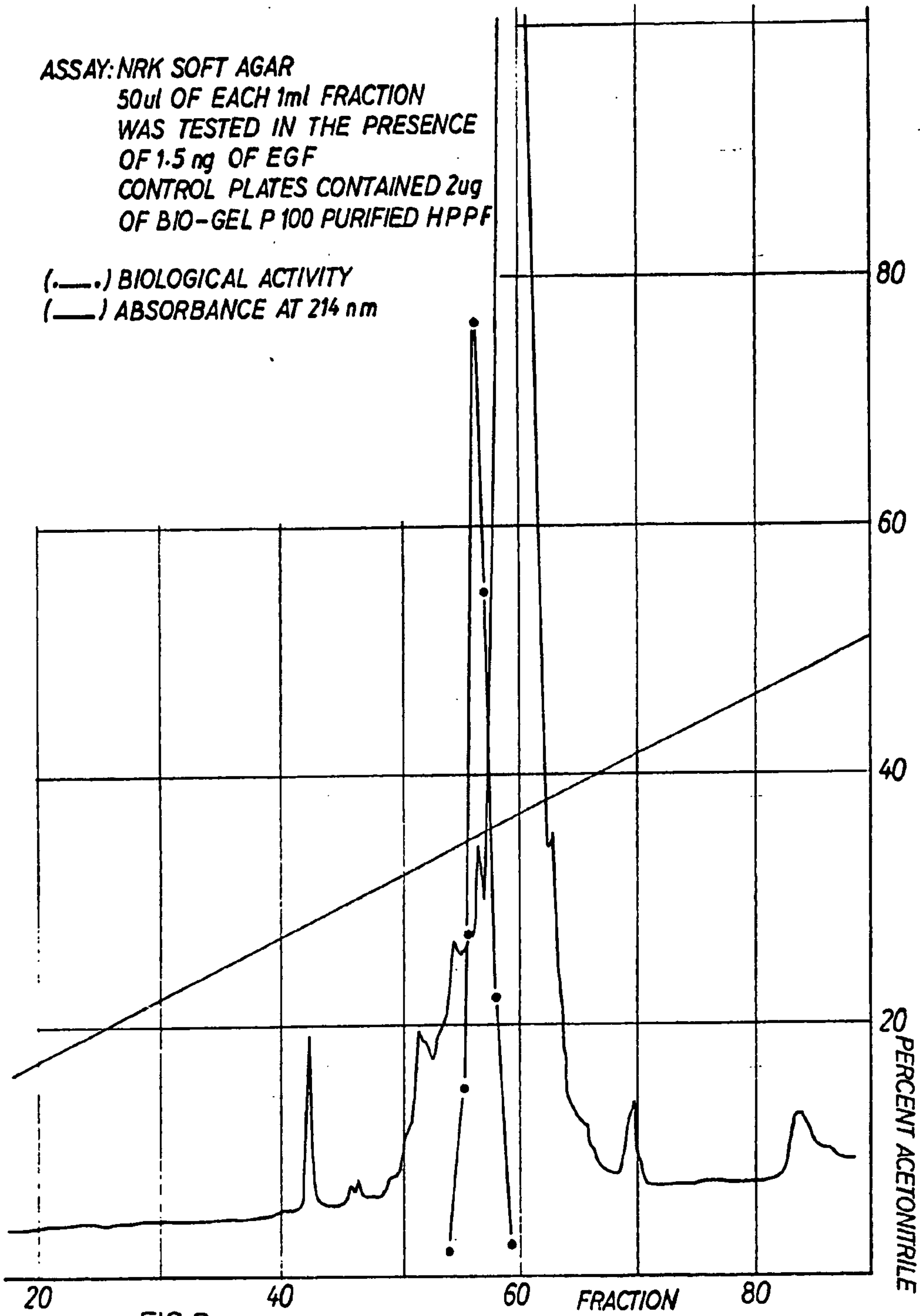
FRACTIONATION OF HPPF BY GEL FILTRATION ON BIOGEL P-100



ASSAY: COLONY FORMATION OF NORMAL RAT KIDNEY CELLS IN 0.3% W/V AGAR IN THE PRESENCE OF 1.5 ng EGF. COLONIES WERE COUNTED AFTER 7 DAYS INCUBATION AT 37°C. 100% WAS EQUIVALENT TO 48% OF THE CELLS FORMING COLONIES.

FIG.1



ASSAY: NRK SOFT AGAR
50ul OF EACH 1ml FRACTION
WAS TESTED IN THE PRESENCE
OF 1·5 ng OF EGF
CONTROL PLATES CONTAINED 2ug
OF BIO-GEL P 100 PURIFIED HPPF
(•—•) BIOLOGICAL ACTIVITY
(——) ABSORBANCE AT 214 nm
80
60
40
20
PERCENT ACETONITRILE
20
40
60
FRACTION
80

FIG. 2

SPECIFICATION

Wound healing compositions

This invention relates to a composition and method for promoting the healing of wounds, including abrasions, lacerations, cuts, burns, etc. to the skin. More particularly, this invention relates to a novel growth factor derived from human platelets, compositions containing the growth factor and to a method for promoting and accelerating the healing of wounds by providing at the site of the wound a mixture of the human platelet proliferation factor and at least one additional biological factor and or activity which are required for cellular growth and repair but which are otherwise unavailable or deficient at the wound site. The invention also relates to products, such as wound dressings, including the therapeutically effective compositions.

Due to the complexity and inherent difficulties in analyzing the wound healing process, no clear understanding of the healing process has emerged from in vivo studies. It is, nevertheless, assumed that the wound healing process in vivo consists of a complex cascade of events occurring in and around the damaged tissue.

However, in recent years considerable progress has been made using cell culture systems and in vivo animal experiments and many factors governing cell growth are known and some of these have been isolated.

For example, the role of fibroblast growth factor (FGF) has been reported by P. Buntrock, et al "Stimulation of wound healing, using brain extract with fibroblast growth factor (FGF) activity I. Quantitative and biochemical studies into formation of granulation tissue", Exp. Path., Vol. 21, 46–53 (1982); "II. Histological and morphometric examination of cells and capillaries", Exp. Path., Vol. 21, 62–67 (1982). See also "The Effect of Fibroblast Growth Factor on the Wound Healing in Monkey Corneas " S. Okisaka, et al, Nippon Ganka Gakkai Zasshi, Vol. 85, No. 9, 1226–36, Sept. 1981 (Jap) (Engl. Abst.) Fibronectin and fibrin are also implicated in the wound healing process as reported by R.A.F. Clark, et al "Fibronectin and Fibrin Provide a Provisional Matrix for Epidermal Cell Migration During Wound Reepithelialization", J. of Inv. Derm. 79: 264–269 (1982); F. Grinnell, "Fibronectin and wound healing "The Americal J. of Dermatopathology, Vol. 4, No. 2, p. 185–188 (Apr 1982); L.A. Repesh, et al "Fibronectin Involvement in Granulation Tissue and Wound Healing in Rabbits", J. of Histochem. and Cytochem, Vol. 30, No. 4, pp. 351–358 (1982); U.S. Patent 4,298,598—O. Schwarz, et al. There have also been several studies relating to epidermal growth factor (EGF), for example M. Niall, et al "The Effect of Epidermal Growth Factor on wound healing in Mice", J. of Surg. Res., Vol. 33, pp. 164–169 (1982); J. W. Thornton, et al "Epidermal growth factor in the healing of second degree burns: a controlled animal study" Burns, Vol 8, No. 3, pp. 156–160 (1981); U.S. Patent 4,287,184—D. M. Young; A. K. C. Li, et al, "Nerve Growth Factor: Acceleration of the rate of wound healing in mice", Proc. Natl. Acad. Sci USA, Vol 77, No. 7, pp. 4379–4381 (July 1980); A. K. C. Li, et al, "Mechanical and humoral factors in wound healing", Br. J. Surg. Vol. 68, pp. 738–743 (1981); K. Leitzel, et al, "Failure of Nerve Growth Factor to Enhance Wound Healing in the Hamster", J. of Neuroscience Research 8:413–417 (1982).

For a general discussion and review of the wound healing process reference can be made to I. K. Cohen, et al "An Update on Wound Healing" Ann. Plast. Surg., Vol 3, pp. 264–272 (Sept. 3, 1979) and S. V. Pollack, "Wound Healing: A Review IV. Systemic Medications Affecting Wound Healing", J. Dermatol. Surg. Oncol. Vol. 8, pp. 667–672 (Aug 8, 1982). Other representative U.S. patent art relating to blood products and extracts as wound coverings and for promoting healing of wounds include U. S. Patents Nos. 1,593,814, 2,912,359, 3,672,954, 3,973,001, 4,177,261 and 4,347,841.

Nevertheless, while much work has been done to study the effects of various cell and blood or plasma derived substances and other chemicals and drugs on the wound healing process, it is clear that much more efficient remedies and treatments would be highly beneficial. Consideration of the basic steps involved in the wound healing process, e.g. collagen metabolism, epithelialization, and contraction, although these can be quite diverse depending on the type and extent of the wound, has not apparently enabled the discovery or selection of the appropriate factors to include in formulations intended for accelerating the wound healing process.

Whether or not the simplified theoretical model of the events occuring in wound healing as set forth by J. K. Cohen and other authors provides an accurate representation of the events occurring during the wound healing process, it is nevertheless clear that a number of biological factors and activators must be present at the wound site if successful and rapid healing is to occur.

It can also be appreciated that wound damage to the vascular network would, at least initially, restrict supply of these essential factors/activators via the blood and, therefore, healing must

depend on the local production of these factors/activators pending repair of the vascular system.

The present invention has been completed on the basis of the discovery of a previously unidentified growth factor derived from human platelets which in combination with at least one additional growth factor and/or activator provide biologically active compositions which upon the topical application to the site of a wound will enhance the rate of repair of the epithelial tissue and the vascular system.

It is accordingly an object of this invention to provide a novel biologically active growth factor derived from human platelets which can be used to promote healing of wounds including repair of the vascular system and epithelial system.

It is a further object of this invention to provide compositions for promoting and accelerating the healing of wounds which include the above mentioned novel human platelet derived growth factor and at least one additional growth factor and/or activator.

A still further object of this invention is to provide a process for treating wounds such as cuts, abrasions, lacerations, burns and the like in order to accelerate the healing process whereby risk of infection time of hospitalization, medical care, loss of working time and similar medical and economic benefits can be achieved.

It is also an object of this invention to provide appropriate products for implementing the above treatment such as wound dressings, "Band-Aid" type dressings, wound irrigation fluids, burn dressings, and the like, which incorporate the biologically active compositions of the present invention.

Briefly, these and other objects of the invention which will become more apparent from the following detailed description of preferred embodiments are accomplished in the first instance by a newly discovered growth factor derived from human platelets which is characterized by being an acid stable and thermostable peptide with an apparent molecular weight in the range of from about 8,000 to 12,500, an isoelectric point in the range of from about 3.5 to 5.5, and sensitivity to disulfide bond reducing agents, and which is eluted from a high pressure liquid chromatography column in the range of from about 35% to about 40% acetonitrile or in the range of from about 25% to 32% n-propanol, in 0.05% trifluoroacetic acid, and by a therapeutically effective wound healing composition of a mixture of said growth factor and at least one biologically active exogenous cellular factor or enzyme selected from cell growth factors, cell attachment factors, and plasminogen activators or inducers thereof.

There is also provided a method for promoting the healing of wounds to the skin which comprises topically applying to the site of the wound an effective amount of a mixture of the above described human platelet derived proliferation factor or activator selected from cell growth factors cell-cell attachment factors and plasminogen activators or inducers thereof.

In a still further aspect the present invention provides dressings and bandages for promoting wound healing in which a flexible substrate is coated or impregnated with the therapeutically effective composition of this invention.

In the accompanying drawings:–

Figure 1 is a graph showing the biological activity in the soft agar colony assay test of the human platelet proliferation factor obtained by gel filtration in combination with epidermal growth factor; and

Figure 2 is a graph showing the light absorbence of the purified human platelet proliferation factor obtained by high pressure liquid chromatography and the biological activity in combination with epidermal growth factor.

On the basis of the in vitro studies and in vivo studies reported in the literature a theoretical model of the wound healing process has been postulated. According to this theoretical model formulated by the inventor, the basic steps of the wound healing process include: (1) mitogenic stimulation leading to DNA synthesis; (2) nutrient supply leading to cell division; (3) collagen or fibromectin mediated cell-cell adhesion leading to tissue formation; (4) endothelial stimulation leading to vascularization; and (5) complete repair. Each of these steps require appropriate biologically available energy sources and appropriate biological cellular and humoral factors and activators. For instance, the mitogenic stimulation requires appropriate growth factors, nutrient supply requires appropriate plasminogen activators (or inducers), cell-cell adhesion requires appropriate attachment factors and endothelial stimulation requires appropriate growth factors.

Moreover, these five steps involve three basic, but essential, activities, namely, (a) induction of plasminogen activators (PA); (b) loss of anchorange dependence; and (c) mitogenesis.

Although not wishing to be found by any particular theory, it is presumed that the wound healing promoting action of the compositions of this invention result from the fact that the combined ingredients, i.e. human platelet proliferation factor and additional cofactor(s), can induce each of these three activities.

The compositions of this invention can be applied to promote healing of any type of wound, such as open granulating wounds and split thickness wounds, and including, for example, skin abrasions, lacerations, punctures, surgical incisions, lesions, bed sores, ulcers, and burns and

more generally where the skin and underlying tissue and/or vascular system have been destroyed or damaged. For example, the wound accelerating technique of this invention can be applied to general surgical or select surgical procedures such as plastic surgery and should be of particular value for burn patients where the risk of infection is particularly high. The invention can also be advantageously applied, for example, in military arenas where promoting wound healing, especially by self administratable compositions and products, e.g. bandages, sprays, etc would be of obvious beneficial value.

It is envisioned that the wound healing compositions can be formulated in any of a number of application forms, dependent on the type of wound, environment, and other conditions. However, three basic forms should be satisfactory to cover most of the situations which can be anticipated. These include:

1. pressurized or pump type aerosol spray in liquid or powder form; for example, a liquid aerosol spray can be dispensed from a conventional atomizer, e.g. pump type bottle, to give fairly large droplet sizes; a powder can be dispensed from an aerosol can with an inert gaseous propellant; the aerosol form would be especially useful where the wound covers a large surface area;

2. liquid preparations in inert or active liquid carriers; useful in irrigation of wounds, especially for surgical procedures, and for application to wound packing materials; can include drop applicator bottles;

3. wet or dry impregnated dressings which can take the form of a Band-Aid type adhesive dressings or a cotton/gauze/cellulose dressing for large wounds; for example, a non-stick dressing which may optionally be provided with a sterile water vial for wetting before being applied.

Additionally, the active ingredients can also be incorporated in aqueous based creams and ointments. In any of these forms, the active ingredients can be used either as a liquid or as a freeze dried powder and can be compounded with a wide range of inert or active carriers, for example, various polymeric hydrogel matrices, and with other additives, such as antibiotics, and other wound healing substances.

One essential ingredient of the therapeutically effective compositions for promoting and accelerating wound healing according to this invention is a newly discovered growth factor derived from human platelets. This new factor has been designated by the inventor as human platelet proliferation factor (HPPF) in order to distinguish this newly discovered substance from a previously known growth factor derived from human platelets which is designated platelet derived growth factor (PDGF). HPPF is characterized by a molecular weight in the range of about 8000 to 12,500 and by an isoelectric point in the range of 3.5 to 5.5, preferably 4.0 to 5.2. PDGF has a molecular weight of about 36,000 and an isoelectric point of about 9.8.

The human platelets from which the human platelet proliferation factor (HPPF) is produced can be fresh, outdated, or platelets which have been frozen, e.g. at about $-70^{\circ}C$. Human platelets are considered outdated three days after collection and are available from any major Blood Banking institute. Best results are obtained with unfrozen fresh or outdated platelet packs. A standard platelet bag contains approximatley 50 grams of platelets and is prepared from 500 milliliters of whole blood collected in about 63 ml of anticoagulant solution, e.g. CPDA-1 solution.

The HPPF is extracted from the platelets using a buffered acid-alcohol extraction solvent. Good results have been obtained using the following buffer solution:

ethanol (95%)	375 ml
HCl (conc)	7.5 ml
phenylmethylsulfonyl fluoride	33 mg
Apoprotin (from Bovine lung)	1 ml

The platelets are mixed with approximately 2 volumes of a buffer solution at approximately $4^{\circ}C$ to extract the biologically active factor inder gentle stirring or low speed homogenization. The resulting mixture is centrifuged at about 5,000–8,000 × g for about 30 minutes. The supernatant is recovered and adjusted to pH of about 5.2 using a weak base such as ammonium hydroxide. The resulting mixture is then again centrifuged at about 5,000–8,000 × g for an additional 30 minutes and the supernatant is again recovered. To this supernatant there is added about 2 volumes of ethanol and 4 volumes of cold ether. The resulting mixture is allowed to stand overnight under deep refrigeration, for example, at about $-70^{\circ}C$. This mixture is again subjected to centrifugation and the resulting precipitate is collected. This precipitate is then resuspended in 1 M acetic acid and dialyzed against 10 volumes of 0.2 M acetic acid. The resulting solution can then be freeze dried to obtain a partially purified preparation of HPPF. This crude preparation exhibits high biological activity in *in vitro* assays when combined with an additional growth factor. For instance, the mixtures of HPPF and the additional cell growth factor can exhibit biological activity in *in vitro* cell culture procedures including stimulation of

normal rat kidney and human diploid foreskin cells to grow in an anchorage independent manner (i.e. to form colonies in soft agar) and enhanced production of plasminogen activator with human diploid lung cells.

The partially purified or crude HPPF preparation can be further purified by gel filtration to enhance the biological activity of the crude HPPF preparation. The further purification by gel filtration can proceed, for example, as follows: the freeze dried HPPF is resuspended in 1 M acetic acid and applied to a 3 × 75 cm glass column containing Bio-Gel P 1.00 presoaked and degassed in 1 M acetic acid. After equilibration in 1 M acetic acid, the sample is applied to the column in a volume of from about 7 to 8 ml. The fractions are collected and assayed for biological activity using a soft agar cloning assay test. The active HPPF fraction elutes in the region of an insulin marker and has an apparent molecular weight of 6,000 to 12, 500, especially 8,000 to 12,500, as confirmed by elution on a Bio-Gel P 10 column carried out by the same procedure. Moreover, HPPF is believed to also exist in a larger form as a dimer or trimer with a molecular weight of from 25,000 to 27,000.

Still further purification of the HPPF fraction collected from the gel filtration column can be achieved using high pressure liquid chromatography (HPLC). In particular, a highly purified HPPF has been obtained using a uBondapak column (Waters Associates). The HPPF can be eluted from the HPLC column using either acetonitrile of n-propanol as the eluent. The first high pressure liquid chromatography step yields the product HPPF at a purity in excess of about 5000-fold. Therapeutically effective compositions for the promotion and acceleration of wound healing in *in vivo* tests have been carried out using the HPPF partially purified by gel filtration. The *in vivo* studies were carried out in rats using the wire mesh wound chamber model (Schilling-Hunt) as described by T. K. Hunt, P Twomey, B. Zederfeldt, and J. E. Dumphy. Am. J. Surg., Vol. 114 302 (1967).

The biologically effective amount of the human platelet proliferation factor in the *in vitro* and *in vivo* applications will depend on such factors as the degree of purification, the co-factor employed therewith, the type of cells being treated *in vitro*, the type and severity of the wound in *in vivo* applications and the like. Generally, for HPPF which has been partially purified by gel filtration biological activity has been demonstrated in terms of cell proliferation capacity in *in vitro* studies in the range of from about 0.1 to about 2.5 micrograms/ml, and in terms of wound healing capacity in the *in vivo* applications in dosage ranges of from about 5 to 50 micrograms per wound chamber in the wire mesh wound chamber model. These activities are increased 20-to 50-fold when the HPPF is further purified by at least one HPLC step.

The second essential component of the therapeutically effective compositions for promoting wound healing according to this invention is at least one co-factor selected from other cell growth factors, cell attachment factors and plasminogen activators and inducers thereof.

Examples of suitable co-factors which can be used in the compositions and methods of this invention together with the human platelet proliferation factor are shown in the following table:

CELL GROWTH FACTORS

Name	Abbreivation	Source
Alpha-thrombin		human plasma
Endothelial Growth Cell Factor	ECGF	bovine tissue
Epidermal Growth Factor (Urogastrone)	EGF	animal tissue human urine
Fibroblast Growth Factor	FGF	animal tissue
Nerve Growth Factor	NGF	animal tissue
Platelet Derived Growth Factor	PDGF	human platelets
Interleukin-1	IL-1	human buffy coats or monocytes
T-Cell Growth Factor (Interleukin-2)	TCGF (IL-2)	human buffy coats or T lymphocytes

CELL ATTACHMENT FACTORS

Name	Source
Human Cellular Fibronectin	human diploid cell cultures
Human Plasma Fibronectin	human plasma

PLASMINOGEN ACTIVATORS/INDUCERS

Name	Source
Urokinase	human urine, human diploid cells, human kidney cells
Streptokinase	bacteria

The preparation of these biological co-factors is well documented in the literature and in addition each of the these materials is commercially available from such sources as the Bethesda Research Laboratories, Inc. (BRL) in Gaithersburg, Maryland; Collaborative Research, Inc. The amount of the co-factor which i required to be used in combination with the human platelet proliferation factor for the *in vitro* and *in vivi* applications will depend on such factors as the type and class of the co-factor, the degree of purity of the co-factor, the type of cells being treated *in vitro*, the type and severity of the wound in *in vivo* applications and the like. Generally, amounts of the co-factor for the *in vitro* applications which exhibit cell proliferation capacity are in the range of from about 1.5 to 5.0 nanograms/ml, while the wound healing capacity in the *in vivo* applications has been exhibited in dosage ranges of the co-factor of from about 1 nanogram (ng) to about 1 microgram (μg) per wound site, depending on purity, and thereby specific activity.

Among the co-factors, the cell growth factors and plasminogen activators and inducers thereof are preferred and particularly epidermal growth factor, fibroblast growth factor, nerve growth factor and platelet derived growth factor, and especially preferably epidermal growth factor.

For example, in the HPPF/EGF system, it has been shown that the EGF component acts on the cells by inducing the production of plasminogen activator in addition to its mitogenic effects. Plasminogen activator (PA) in turn converts serum plasminogen to the active protease plasmin. Plasmin, along with other enzymes, digests blood proteins dead tissue, blood clots, etc. to yield amino acids which constitute the essential nutrients for cell growth. This role of plasminogen activators is suggested by Michael Gronow and Rudolph Bliem in "Production of human plasminogen activators by cell culture", Trends in Biotechnology, Vol. 1, No. 1, pages 26–29, (1983). Accordingly, this mode of action induces cleansing of the wound in addition to promoting wound healing.

By whatever mechanism the compositions of this invention exert their wound healing capacity, it is a particularly advantageous feature of this invention that the biologically active materials are non-toxic and are normal cellular products. Moreover, the new cells which are produced in the presence of the therapeutic compositions of this invention are completely normal and no chromosomal or structural abnormalities have been observed.

In addition ito the human platelet proliferation factor and the additional essential co-factor or co-factors, the compositions of the present invention may further include biologically available energy sources, such as sodium pyruvate, sugars, etc, antibiotics, such as thermostable broad spectrum antibiotics, e.g. Gentamicin Sulfate; and other ingredients known to be useful in promoting wound healing, for example, vitamins, such as Vitamin A, E and C, zinc compounds, such as zinc sulfate, diphenylhydantoin, and the like.

A preferred composition for promoting wound healing according to this invention, which would be suitable for wounds up to about 1 square inch, includes:

human platelet proliferation factor	5 to 500 μg
epidermal growth factor	5 to 50 ng
carbon (energy) source	20 to 500 μg
antibiotic	2 to 100 μg

*based on purification on Bio-Gel P 100 column, for HPPF purified by high pressure liquid chromatography the amount can be reduced by a factor of from about 20 to about 50, i.e. 0.1

to 25 μg.

For larger wound sites, the amounts of each of the individual ingredients can be increased proportionately.

When the compositions are coated on or impregnated in a flexible substrate to form a wound dressing it is preferred to apply a new dressing each day. However, where such change of dressing is contraindicated a larger initial dose of the wound healing promoting composition can be applied.

EXAMPLE 1

Extraction of crude HPPF

Fresh human platelets are isolated from one unit of blood yielding about 48.7 grams wet weight of platelets. The platelets are suspended in 2 volumes of a buffer formed by adding 2 volumes of a buffer formed by adding 33 mg phenylmethylsulfonyl fluoride and 1 ml apoprotin isolated from bovine lung to a mixture of 7.5 ml concentrated HCl and 375 ml ethanol gently homogenized at about 4°C. The resulting mixture is centrifuged at 5000 × g for 30 minutes and the supernatant is collected and its pH is adjusted to 5.2 with ammonium hydroxide. The resulting supernatant is re-centrifuged at 5000 × g for another 30 minutes and the supernatant is then mixed with 2 vol/vol of ethanol and 4 vol/vol of cold ether and the resulting mixture is refrigerated at a temperature of about −70°C overnight. The resulting precipitate is collected by centrifugation, resuspended in 1M acetic acid and dialyzed against 10 volumes of 0.2M acetic acid. The resulting product is lyophilized to dryness. The freeze dried product containing the crude human platelet proliferation factor is analyzed and contains about 247.5 mg protein.

EXAMPLE 2

Purification of HPPF

The lyophilized crude HPPF (247 mg) from Example 1 is reconstituted in 7.5 ml of 1M acetic acid and applied to a 3 × 75 cm glass column containing Bio-Gel P100 molecular sieve previously equilibrated with 1M acetic acid. 120 fractions each having a volume of 3.5 ml are collected from the column. The column is calibrated using appropriate markers for determining the approximate molecular weight of the active fraction containing the purified HPPF. For this purpose, carbonic anhydrase marker, cytochrome C marker and insulin marker are used.

From each fraction a 50 microliter sample is taken and transferred into a small tube and lyophilized for testing in a soft agar cloning assay. The results are shown in Fig. 1. In view of the molecular weight of insulin of about 6,000 and the molecular weight of cytochrome C of about 13,500, the molecular weight of the HPPF in the fractions showing peak activity (fractions 90, 91 and 92) is estimated to be in the range of from about 8,000 to 12,500.

Furthermore, the peak fractions 90, 91 and 92 are pooled together and the protein concentration is determined by the Lowry method against an albumin standard and found to contain 1.2 mg of protein.

The soft agar cloning assay is carried out as follows: a 5% agar solution is prepared by mixing 50 grams of Noble agar (Difco) in 100 ml distilled water. After boiling to dissolve the agar, the solution is distributed in 20 ml aliquots and autoclaved at 115°C for 15 minutes. The test procedure is carried out with low passage normal rat kidney cells from cultures which are non-confluent and which are growing vigorously at the time of use.

An 0.5% agar medium is prepared by melting the previously prepared 5% agar in a boiling water bath at which time it is mixed well and boiled for a further 5 minutes. 2 millimeters of the hot agar are added to an 18 milliliter aliquot of Dulbecco's modified Eagles medium with high glucose or with pyruvate and supplemented with 10% v/v pretested calf or foetal calf serum, 100 iu/μg per ml of Penicillin and Streptomycin and 200 mM of L-glutamine (DMEM) and mixed thoroughly.

1 ml aliquots of the 0.5% agar are pipetted into 1 inch petri dishes and the agar is allowed to solidify for 15 minutes.

The test samples from each of the fractions obtained from the Bio-Gel P100 column are mixed with 1.5 nanograms of Epidermal Growth Factor (EGF) prior to lyophilization in the small test tubes. The samples are substantially isotonic and at physiological pH.

To prepare the normal rat kidney cells for the assay, the cells are detached from the surface in which they are grown by treatment with 0.05% trypsin in 0.01 M ethylene diamine tetracetic acid (EDTA) in balanced salt solution and the cells are then resuspended in DMEM. The cell suspension is diluted to 3×10^4 cells per ml on the basis of haemacytometer count. To each sample tube 0.3 ml of cells are added and mixed quickly.

Then to each test sample tube 0.75 ml of 0.5% agar at 46°C is added. Each tube is then gently mixed on a vortex mixer and poured onto the agar base layer in a single petri dish. The tubes should be handled only a few at a time to prevent agar solidification. The plates are then incubated at 37°C in a well humidified incubator with 5% CO_2 and air.

The petri dishes are examined microscopically at intervals of 2–3 days. The number of colonies containing at least 50 cells are counted. If the test is to be continued beyond 5 days a further 1 ml of 0.3% agar is added to prevent drying.

In Fig. 1, the number of colonies are counted after 7 days incubation and 100% activity is equivalent to 302 colonies each with more than 50 cells or 48% of the cells forming colonies. In the absence of added factors or with EGF alone no growth is observed.

EXAMPLE 3
High Pressure Liquid Chromatography (HPLC) of HPPF

Fractions 90, 91 and 92 from the Bio-gel P 100 column obtained in Example 2 are lyophilized and resuspended in 0.5% v/v trifluoroacetic acid (TFA) in water and adjusted to pH 2.0. This sample is then injected into a μ Bondapak TM/C18 column (Water's Associates) previously equilibrated with 0.05% v/v TFA. The fractions are then eluted from the column with a linear gradient of acetonitrile in 0.045% TFA at a flow rate of 1 ml per minute and collected in fractions of 1 ml. The light absorbence of each fraction at 214 nm is measured and the results are shown in Fig. 2.

Also illustrated in Fig. 2 are the results obtained when samples of each of the fractions from the HPLC to which have been added 1.5 ng of EGF are tested in the soft agar assay system previously described against a control preparation of HPPF purified on the Bio-Gel P 100 column. In this experiment, the control gave 300 colonies of greater than 50 cells and HPLC fractions are expressed as a percentage of this value, i.e. the maximum peak corresponds to 76.5% of the control.

The pooled peak fractions 57 and 58 contain approximately 2 micrograms of protein. When these fractions are subjected to standard polyacrylamide gel electrophoresis (PAGE) analysis only 3 to 5 peptide bands are observed in the molecular weight range of 8,000 to 12,500 Daltons.

Similar results are obtained in the HPLC system using a 0–60% linear gradient of n-propanol in place of acetonitrile except that the fractions showing peak activity in the soft agar cloning assay are eluted from the μ Bondpak C18 column in the range of 28–32% n-propanol.

The purification steps by acid alcohol extraction gel partition separation, and high pressure liquid chromatography result in the following yields of active substance (HPPF):

Purification Step	Yield HPPF (units/μg protein) [1 unit = 50 colonies/field]
Extraction	12
P 100 column	560
HPLC column	15,612

EXAMPLE 4
Measurement of Physicochemical Properties of HPPF

1. Protease Sensitivity

HPPF purified by gel filtration (Example 2) is lyophilized and reconstituted in 0.1 M NH_4HCO_3. 10μg/ml of 2 × crystallized trypsin (Sigma) is then added and the sample is incubated at 37°C for two hours. Soybean trypsin inhibitor is then added to 70 μg/ml of the HPPF sample. The samples are then tested in parallel with a control without trypsin for colony formation in soft agar according to the previously described procedure with the results shown in Table 2. All tests are carried out in the presence of 1 ng/ml of EGF.

TABLE 2

	Protease Sensitivity	
Group	Plate No.	Colonies/Field (50 cells)
Control	1	>59
	2	>59
	3	>59
Trypsin	4	None
	5	None
	6	None

2. Acid Stability

Self evident in view of ability to purify in 1M acetic acid.

3. Thermostability

HPPF as in 1 above is exposed at 56°C in 0.1M NH_4CO_3 for 1 hour and then assayed in parallel with an untreated control in the presence of 1 ng/ml of EGF. The results are shown in Table 3.

TABLE 3

Thermostability		
Group	Plate No.	Colonies/Field (50 cells)
Control	1	>59
	2	>59
	3	>59
56°C 1 hour	4	53
	5	>59
	6	54

4. Molecular Weight

The molecular weight is in the range of 8,000 to 12,500 as determined by gel filtration on Bio-Gel P100 (see Example 2 and Fig. 1). In addition, when gel filtration is carried out on a Bio-Gel P10 column HPPF activity elutes between the insulin (6,000 MW) and cytochrome C (13,500 MW) markers. From a semi logarithmic plot of MW against fraction number, peak HPPF activity eluted at an apparent molecular weight of between 8,000 and 12,000. Accordingly, the preferred apparent molecular weight range of the purified HPPF is in the range of 8,000 to 12,000.

5. Iso-Electric Point (pI)

Peak fractions from a P 100 column are lyophilized from 1 M acetic acid and reconstituted in 1.2 ml of distilled water. This sample is then applied to a flat bed iso-electric focusing apparatus (LKB Multiphor). After operation for 6 hours at 4°C and a constant power of 8 W, the gel is separated in 22 fractions and the pH is measured. All fractions are then eluted with 1 M acetic acid, dialyzed against 100 volumes of 0.2 M acetic acid and lyophilized. Samples are then tested for the cloning of NRK cells in soft agar in the presence of 1 ng/ml of EGF. Results of this study are shown in Table 4:

TABLE 4

ISO-Electric Point		
Fraction	pH	Colonies/Field
2	4	95,
4	4.5	25, 21
6	5.2	11, 2
8	5.7	3, >1
10	6.1	>1, >1
12	6.4	>1, >1
14	6.8	>1, >1
16	7.5	>1, >1
18	8.3	>1, >1
20	9.2	>1, >1
22	9.6	>1, >1

From the results of the above table the pI of HPPF is taken to be in the range of 3.5 to 5.5, especially from 4 to 5.2.

6. Dithiothreitol Sensitivity

The procedure is generally as in 1 and 3 above. A control sample and a test sample containing 0.1 M dithiothreitol are incubated at 56°C for 1 hour. The samples are then dialyzed against 0.2 M acetic acid (extensively), lyophilized and tested for activity in the soft agar cloning assay. The results are shown in Table 5.

TABLE 5

Dithiothreitol Sensitivity		
Group	Plate No.	Colonies/Field (50 cells)
Control	1	51
	2	54
	3	49
Dithiothreitol	4	1
	5	4
	6	0

7. HPLC Elution

The elution characteristics from the HPLC system (Example 3) reflect the unique properties of HPPF, i.e. elutes from the C18 TM u Bondapak column (Waters Associates at 37–42% acetonitrile or 28–32% n propanol where both eluants are used in the form of 0–60% linear gradients with trifluoroacetic acid.

EXAMPLE 5

Measurement of Biological Properties of HPPF

Replicate cultures of MRC-5 human diploid lung cells are washed with serum free 199 medium* and overlaid with the same medium containing 0.5% foetal bovine serum at 0.3 ml/cm^2. Partially purified HPPF from Example 2 (peak fraction 90, 91 and 92) (10 μg/ml) and EGF (1.5 ng/ml) are then added to the test cultures. After incubation at 37°C for the time indicated in Table 6, samples are removed for assay of plasminogen activator by the method of Barnett and Baron (PSEBM *102*; 308–311). Results are expressed as international reference units per milliliter as Urokinase and are shown in Table 6.

TABLE 6

Production of plasminogen activator by MRC-5 cells in response to HPPF/EGF

Experiment No.	Incubation Time Days	Plasminogen Activator iu/ml Control	HPGF/EGF	Fold Increase
1	2	2.5	6.7	2.7
	4	10	40	4
2	7	22	105	4.8
3	7	18	63	3.5

*Synthetic cell culture medium consisting of amino acids vitamins, etc., in a balanced salt solution (available from Gibco); used without serum supplement.

EXAMPLE 6

This example demonstrates the promotion of wound healing in the rat wound healing model by a mixture of HPPF and EGF. The wire mesh wound healing model used is that of Schilling-Hunt, which is described in detail in a paper by T. K. Hunt, P. Twomey, B. Zederfeldt, and J. E. Dumphy, Am. J. Surg., Vol. 114, p. 302 (1967).

Male and female rats weighing from 350 to 500 grams are used. Wound chambers measuring 1.5 cm in length by 0.5 cm radius are implanted in the back region of the test rats. The wound chambers are injected subcutaneously every day for 8 days with the materials indicated in Table 7. On day 9. the chambers are additionally injected with radioactively labelled precursors as shown in Table 7. After 6 hours from the last injection, the chambers are removed and TCA precipitatable protein and radioactivity are determined. Duplicate rats are used for each experiment. Protein is determined by the Lowry method. The dosages used in the injections include 20 μg human serum albumin (HSA), 50 ng EGF, and 20 μg HPPF (purified by gel filtration). The results are shown in Table 7.

TABLE 7

Effect of HPPF and EGF on the Incorporation of Radiolabelled Precursors in the Wound Healing Model

Precursor	No. of Experiments	Mean Fold Increase in Incorporation of Label per mgm o f Protein relative to Albumin Control (Range) EGF + HSA	EGF + HPPF
^3H Thymidine	5	1.30(0.95–1.79)	3.89 (2.43–5.29)
^3H Leucine	5	2.01 (0.9603.35)	6.07 (4.69–7.56)
^{14}C Hydroxy Proline	3	Not Tested	7.22 (2.42–13.9)

From the data in Table 7, it can be appreciated that the mixture of HPPF with EGF accelerates healing (based on total protein and collagen at the end of the test period, and confirmed by increased uptake of labelled thymidine, leucine and hydroxyproline) by 2 to 3 fold in a period of 9 to 10 days.

CLAIMS

1. A method for promoting the healing of wounds to the skin which comprises topically applying to the site of the wound an effective amount of a composition comprising a mixture of human platelet proliferation factor and at least one additional biological factor selected from the group consisting of cell growth factors, cell attachment factors and plasminogen activators, or inducers thereof said human platelet proliferation factor being an acid stable, thermostable peptide with an apparent molecular weight in the range of about 8,000 to about 12,500 and an isoelectric point in the range of from about 3.5 to about 5.5, and which is eluted from a high pressure liquid chromatography column in the range of about 32% to about 40% acetonitrile in 0.05% trifluoroacetic acid or in the range of about 25% to 32% n-propanol in 0.05% trifluoroacetic acid.

2. The method of claim 1 wherein said mixture comprises purified human platelet profileration factor and a cell growth factor selected from the group consisting of alpha-thrombin, endothelial cell growth factor, epidermal growth factor, fibroblast growth factor, nerve growth factor, platelet derived growth factor, interleukin-1, interleukin-2 and mixtures thereof.

3. The method of claim 2 wherein said cell growth factor comprises epidermal growth factor.

4. The method of claim 3 wherein said mixture comprises from about 0.002 to about 0.125 microgram of purified human platelet proliferation factor and from about 1.5 to about 5.0 nanograms of epidermal growth factor.

5. Human platelet proliferation factor which is characterized as being an acid stable, thermostable peptide with an apparent molecular weight in the range of 8,000 to 12,500 an isoelectric point in the range of from about 3.5 to about 5,5, sensitive to dithiothreitol, and which is eluted from a high pressure liquid chromatography column in the range of about 32% to about 40% acetonitrile or in the range of about 25% to 32% n-propanol, in 0.05% trifluoroacetic acid, and which, in admixture with epidermal growth factor exhibits the ability, *in vitro,* to stimulate normal rat kidney and human diploid foreskin cells to grow in an anchorage independent manner, and induces enhanced production of plasminogen activator by human diploid lung cells, and, *in vivo,* promotes wound healing.

6. The human platelet proliferation factor according to claim 5 which is extracted from fresh or outdated human platelets by extraction with a mineral acid-ethanol buffer solution.

7. A composition effective for promoting the healing of wounds which comprises the human platelet proliferation factor of claim 5 and at least one additional co-factor selected from the group consisting of cell growth factors, cell attachment factors and plasminogen activators.

8. The composition of claim 7 wherein the additional co-factor comprises epidermal growth factor.

9. The composition of claim 8 which comprises human platelet proliferation factor and epidermal growth factor at a weight ratio in the range of from about 2:1 to about 500:1.

10. The composition of claim 7 in the form of a freeze dried powder.

11. The composition of claim 7 in the form of a liquid.

12. A wound dressing comprising a flexible substrate impregnated with the composition of claim 7.

13. A wound dressing comprising a flexible substrate coated with the composition of claim

7.

14. An aerosol spray for promoting healing of wounds comprising the composition of claim 7 and a gaseous propellant.

15. The features herein described, or their equivalents, in any patentably novel selection.

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